Yale University
EliScholar – A Digital Platform for Scholarly Publishing at Yale

Yale Medicine Thesis Digital Library | School of Medicine

1991

DNA probes detect Theileria parva in the salivary glands of Rhipicephalus appendiculatus ticks

Philip Peihai Chen
Yale University

Follow this and additional works at: http://elischolar.library.yale.edu/ymtdl

Recommended Citation
Chen, Philip Peihai, "DNA probes detect Theileria parva in the salivary glands of Rhipicephalus appendiculatus ticks" (1991). *Yale Medicine Thesis Digital Library*. 2457.
http://elischolar.library.yale.edu/ymtdl/2457

This Open Access Thesis is brought to you for free and open access by the School of Medicine at EliScholar – A Digital Platform for Scholarly Publishing at Yale. It has been accepted for inclusion in Yale Medicine Thesis Digital Library by an authorized administrator of EliScholar – A Digital Platform for Scholarly Publishing at Yale. For more information, please contact elischolar@yale.edu.

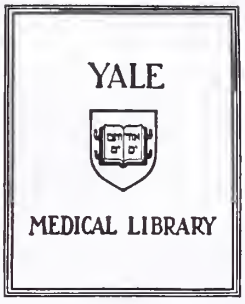
YALE MEDICAL LIBRARY
3 9002 08676 1401

DNA probes detect Theileria parva in the salivary glands of Rhipicephalus appendiculatus ticks

Philip Peihai Chen

Yale University

1991


YALE
MEDICAL LIBRARY

Permission for photocoping or microfilming of "DNA PROBES TO DETECT THEILERIA PARVA IN THE SALIVARY GLANDS OF R. APPENDICULATUS TICKS."

(Title of thesis)

for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Signature of Author

2/10/91

Date


Digitized by the Internet Archive
in 2017 with funding from
The National Endowment for the Humanities and the Arcadia Fund

https://archive.org/details/dnaprobesdetectt00chen

DNA probes detect *Theileria parva* in the salivary glands of *Rhipicephalus appendiculatus* ticks

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Philip Peihai Chen

1991

Med WC
T13
M12
5906

YMLJUL 30 '91

Acknowledgements

I thank Mr. Stephen Mwaura and the staff of the International Laboratory for Research on Animal Diseases (ILRAD) Tick Unit for tick dissection and salivary gland examination. I received a Downs International Health Travel Fellowship from the Committee on International Health of the Yale University School of Medicine, and ILRAD provided further financial support. Dr. Patricia A. Conrad of ILRAD generously supplied the *T. parva*-specific DNA probes and guided me through the project. Dr. Onesmo K. ole MoiYoi of ILRAD allowed me use of his lab space and provided timely advice. Dr. Thomas T. Dolan of ILRAD supplied lab space and intellectual support. Dr. Curtis Patton of the Yale University School of Medicine, Department of Epidemiology and Public Health was instrumental in arranging my visit to ILRAD, and served as my thesis advisor. My sincere thanks to all.

Abstract

DNA PROBES DETECT *THEILERIA PARVA* IN THE SALIVARY GLANDS OF *RHIPICEPHALUS APPENDICULATUS* TICKS. Philip P. Chen, Patricia A. Conrad. International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya. (Sponsored by Curtis L. Patton, Department of Epidemiology and Public Health, Yale University School of Medicine).

Intracellular parasitic protozoa of the genus *Theileria* cause East Coast fever in cattle, via the tick vector *Rhipicephalus appendiculatus*. Current immunization methods confer resistance to homologous strains of *Theileria spp.* Studies to determine cross-protectivity have led to the development of DNA probes specific for *T. parva.* In the present study, two DNA probes (pgTpM-23 and lgTpM-58) were hybridized separately to parasite DNA in samples prepared from the salivary glands of infected ticks. Each of six different parasite stocks was used to infect 25-200 adult ticks. One salivary gland from each tick was processed for DNA hybridization, while the other was stained and examined by light microscopy to determine the number of infected acini. Correlation between the two methods of detecting infected acini ranged from 84-100% with pgTpM-23, depending on the stock of *T. parva*. Correlation with lgTpM-58 ranged from 90-100%. The discrepancy was within the range expected, based on a separate study in which 13% of 62 infected ticks had only one infected salivary gland. Both probes appeared to be able to detect a single infected salivary gland acinus, and neither probe hybridized to salivary glands from uninfected ticks. Probe pgTpM-23 had

a specificity of 97.0%, a sensitivity of 94.1%, and a positive predictive value of 96% in the tick population studied, under optimal conditions. Probe lgTpM-58 had a specificity of 92.1%, a sensitivity of 91.6%, and a positive predictive value of 98% in the tick population studied, under optimal conditions, but displayed non-specific hybridization to uninfected controls after extended periods of autoradiography.

Table of Contents

Introduction.....1

Materials and Methods.....4

Results.....10

Discussion.....13

References.....18

Figures.....25

Tables.....35

List of Figures and Tables

Fig. 1. Distribution of East Coast fever.....25

Fig. 2. Life cycle of *Theileria*.....26

Fig. 3. Electron micrograph of bovine lymphocyte infected with *T. parva* schizonts.....27

Fig. 4. Electron micrograph of piroplasm stage of *T. parva*.....27

Fig. 5. *R. appendiculatus* (brown ear tick) dissection.....28

Fig. 6. Development of DNA probes specific for *T. parva*.....29

Fig. 7. Light micrograph of salivary gland acini stained with Fuelgen's stain.....30

Fig. 8. Autoradiograph of hybridization of clone pgTpM-23 to *T. p. parva* (Uganda) and *T. p. parva* (Muguga).....31

Fig. 9. Autoradiograph of hybridization of clone pgTpM-23 to *T. p. parva* (Marikebuni) and *T. p. parva* (Muguga).....32

Fig. 10. Autoradiograph of hybridization of clone lgTpM-58 to *T. p. parva* (Marikebuni).....33

Table 1. Correlation between visual examination and hybridization of probes pgTpM-23 and lgTpM-58 in detection of *Theileria parva* in tick salivary glands.....35

Table 2. Results of hybridization of probe pgTpM-23 to tick salivary glands.....36

Table 3. Results of hybridization of probe lgTpM-58 to tick salivary glands.....37

Table 4. Summary of comparison of salivary gland infection rates.....38

Table 5. Sample of results from visual examination of stained salivary gland acini.....38

Table 6. Statistical analysis of hybridization results with *T. parva*-specific DNA probes and tick salivary glands.....39

Introduction

Intracellular parasitic protozoa of the genus *Theileria* cause disease of varying severity in cattle, sheep, and other domestic and wild animals in tropical and sub-tropical regions of Africa, Asia, the Middle East, and North America. An especially virulent form of theileriosis called East Coast fever (ECF) is caused by *Theileria parva* and is transmitted by the brown ear tick, *Rhipicephalus appendiculatus.* ECF is endemic in cattle populations of eleven East and Central African countries (Fig. 1). Classical ECF is caused by *T. parva parva.* Rhodesian malignant theileriosis, a distinct disease syndrome of cattle in Zimbabwe and neighboring countries, is caused by *T. parva bovis* (Uilenberg, 1981). The African buffalo (*Syncercus caffer*) can be infected but is much less susceptible to ECF. Although they are not domesticated and therefore are not in continuous close contact with cattle, buffalo act as a reservoir for transmission of infection by ticks (Conrad *et al.*, 1987a; Irvin, 1987), and parasites derived from buffalo are known as *T. parva lawrencei*, a subspecies clinically and epidemiologically distinct from *T. parva parva*, and causing Corridor disease (Uilenberg, 1981). The three subspecies are morphologically and serologically indistinguishable.

The theilerial life-cycle is well-adapted to disease transmission (Fig. 2). (Reviewed by Barnett, 1977, and Melhorn & Schein, 1984). The infective forms of the parasite, sporozoites, develop in tick salivary glands and are passed to cattle during feeding. Sporozoites invade host lymphocytes, where they develop into schizonts.

Infected lymphocytes become enlarged and transform into lymphoblasts, which then multiply synchronously with the parasites, resulting in a rapidly expanding population of parasitized cells (Fig. 3). Massive infection of the lymphoid system, clinically manifested by fever, diarrhea, and lymphadenopathy, often leads to death due to interstitial pneumonia and pulmonary edema within two to four weeks in susceptible cattle populations. An estimated 500,000 cattle die each year in Africa due to ECF (Miller, 1977). The disease is a significant obstacle to human and agricultural development in the region.

Merozoites develop during the later stages of infection and are released from the infected lymphocytes, whereupon they invade host erythrocytes and become the piroplasm stage of the organism (Fig. 4); this stage in turn infects ticks that feed on the blood of infected hosts. Some piroplasms differentiate at this time into gametocytes. In the tick gut, gametocytes mature into gametes which unite to form zygotes. Zygotes penetrate the gut epithelium and form ookinetes. The ookinetes migrate to the salivary gland via hemolymph, and there form sporoblasts in the E cell of the Type III salivary gland acini. Upon the ingestion of a blood meal by the tick, the sporoblast increases in size and complexity, forming a multinucleate syncitium until it fragments by cytoplasmic fission into thousands of sporozoites within the salivary gland acinus (Fawcett, Buscher & Doxsey, 1982).

Current control of ECF is based primarily on expensive and sometimes unreliable measures to kill ticks by dipping or spraying cattle with acaricides. The only effective method of immunization of naive cattle now available is an infection and treatment

protocol, in which the animal is deliberately infected with a cocktail containing sporozoite forms from several isolates of the parasite. The animal is then treated with a long-acting tetracycline, usually oxytetracycline hydrochloride (Radley *et al.*, 1975). If this treatment fails, parvaquone, a naphthoquinone (Dolan *et al.*, 1984) or halofuginone hydrobromide, an anticoccidial (Dolan, 1987) may be used. This protocol confers resistance to homologous strains of *T. parva*, but susceptibility to heterologous strains remains (Irvin *et al.*, 1983). Therefore, studies to characterize the strains of *T. parva* and to determine which strains are cross-protective are important. DNA probes have been used increasingly to identify both pathogenic and non-pathogenic species, strains, and clones of parasites causing leishmaniasis (Wirth & Pratt, 1982; Jackson *et al.*, 1986; Rogers, Burnheim & Wirth, 1988; van Eys *et al.*,1989), filariasis (McReynolds, DeSimone & Williams, 1986; Sim *et al.*, 1986), babesiosis (McLaughlin, Edlind & Ihler, 1986), malaria (Franzen *et al.*, 1984; Bhasin *et al.*, 1985; Pollack *et al.*, 1985; Barker *et al.*, 1986), trypanosomiasis (Morel *et al.*, 1980; Kukla *et al.*, 1987; Sturm *et al.*, 1989), and onchocerciasis (Perler & Karam, 1986; Erttmann *et al.*, 1987), and to detect these parasites within their vectors. DNA probes capable of differentiating stocks, isolates, and clones of *T. parva* based on restriction fragment length polymorphisms have been developed (Conrad *et al.*, 1987b; Conrad *et al.*, 1989; Allsopp & Allsopp, 1987; Allsopp *et al.*, 1989). The present study describes the results of hybridization of DNA probes to sporozoites within the salivary glands of ticks infected with various laboratory stocks of *T. parva*. Specific detection of parasite DNA within the vector could prove useful in development of a diagnostic test for field epidemiology and immunization studies.

Materials and Methods

The nomenclature used throughout this thesis to describe *Theileria* has been adopted from the World Health Organization (WHO) system of trypanosome nomenclature (WHO, 1978) as modified by Irvin *et al.*, 1983:

Isolate. Viable organisms isolated on a single occasion from a field sample into experimental hosts or culture systems, or by direct preparation of a stabilate.

Stock. All the populations of a parasite derived from an isolate without any implication of homogeneity or characterization. Populations comprising a single stock thus include cell lines and tick stabilates, and subsequent parasite populations derived from them.

Strain. A population of homogeneous organisms possessing a set of defined characters. Unambiguous characterization of a strain can be assured only if the population of organisms was initiated from a parasite clone, i.e. derived from a single parasite.

Stabilate. A sample of organisms preserved alive (usually in replicate) on a single occasion.

Laboratory isolates of sporozoites, maintained as stabilates in liquid nitrogen, from five stocks of *T. parva parva* and one stock of *T. parva bovis* isolated in East Africa (Table 1) were injected subcutaneously to infect calves six to twelve months old

(Conrad *et al.*, 1989). Nymphal *R. appendiculatus* ticks were fed on the infected calves and then held at 28°C in CO_2 incubators until needed. After moulting, the ticks were fed on uninfected rabbits for 3-4 days before dissection, to stimulate sporozoite maturation in the salivary glands. Not all ticks that are fed on infected animals become infected themselves, and capable of parasite transmission; infection rates are strain dependent, ranging from 1.5% to 80%. Most stocks had high rates of infection. In the present study, the number of ticks used from each stock is based on the predicted infection rates of the ticks, to ensure that at least some of those ticks tested would indeed be infected. For each of the five *T. p. parva* stocks, (Muguga, Uganda, Marikebuni, Zanzibar South, and Mariakani), 25-60 ticks were tested. For the *T. p. bovis* Boleni stock 200 ticks were evaluated using the pgTpM-23 probe because the tick infection rate with this stock was known from previous experiments to be exceptionally low (less than 5%). The lgTpM-58 probe was not tested on ticks infected with the Boleni stock, due to the low tick infection rate.

Each tick was pinned on a blackened wax disc submerged in a Petri dish containing unbuffered normal saline, pH 7.0 (Fig. 5). The two salivary glands were carefully removed under dissecting microscopes using no.15-bladed scalpels, fine forceps, and straight pins; this specialized procedure was carried out by ILRAD Tick Unit technicians in the vast majority of experiments. One gland was placed on a numbered glass slide for subsequent microscope examination; the second gland was placed in a corresponding, numbered well of a 96-well microculture plate. Each well

contained 300 µl of unbuffered normal saline, pH 7.0. Notation of microscope slide position and microculture plate number allowed comparison between the results of microscopy and the results from hybridization, for each tick. The entire second gland was spread and smeared, using forceps, onto an 8 cm. x 12 cm. piece of nylon filter paper (Hybond-N, Amersham) prewetted in 50 mM Tris, 0.25 M EDTA, 0.75 M NaCl. Each filter held 12 to 110 smears, depending on the experiment. Salivary glands from uninfected ticks served as negative controls, and glands from tick batches known from past experiments to be heavily infected were used as positive controls.

Salivary glands on each filter were lysed with a 10% sodium dodecyl sulfate (SDS) solution for ten minutes, followed by a five minute drying period on Whatman no. 3 filter paper. The DNA was denatured in 1.5M NaCl and 0.5 M NaOH for ten minutes, neutralized two times for ten minutes each in 1.5M NaCl and 0.5 M Tris·HCl (pH 7.5), and blot-dried on filter paper. A four-minute exposure to UV light fixed the DNA on the nylon filter. All filters were incubated at 65°C for 5-18 hours in sealed plastic bags containing hybridization fluid [4x saline sodium citrate (SSC) (1x SSC = 150 mM NaCl, 15 mM sodium citrate), 10x Denhardts's solution, 0.1% SDS, 0.1% sodium pyrophosphate].

The probes used were clones from *T. p. parva* (Muguga) containing repetitive DNA sequences. Clone pgTpM-23 contained a 2.3 kb insert in plasmid vector pUC19; clone lgTpM-58 contained sequences with internal EcoR1 sites yielding insert fragments of 0.8, 1.0, 1.9 and 2.1 kb in bacteriophage vector λ gt11. The development

of these probes has been previously published (Conrad et al., 1987b). To summarize the process (Fig. 6), *Theileria* piroplasm DNA obtained from infected cattle erythrocytes was purifed by RNAse treatment and phenol:chloroform extraction, sheared by 300 passages through a 19-gauge syringe needle, and prepped for EcoRI endonuclease digestion. The resulting DNA fragments were inserted into λ gt11 arms and packaged into phage particles to create a genomic expression library of *T. p. parva* (Muguga) DNA. The genomic library phage was amplified by plating on *E. coli* Y1090 r- cells, and duplicate plaque transfers onto nitrocellulose filters were made from the resulting plaques. After appropriate preparation, the filters were hybridized to radiolabelled total bovine lymphocyte DNA or to total *T. p. parva* (Muguga) piroplasm DNA. Plaques that hybridized most strongly under low C_0t conditions (low DNA concentration, short hybridization time) to *T. parva* DNA, but not to bovine lymphocyte DNA, were assumed to contain high amounts of repetitive DNA sequences. Repetitive DNA is thought to evolve rapidly and has been shown to have a high amount of species- and strain-specificity (Jelinek & Schmid, 1982). The plaques selected were purified twice using the same *in situ* plaque hybridization procedure described above, cloned by the plaque lysate method (Maniatis, Fritsch & Sambrook, 1982), and radiolabelled for use as probes on Southern blots of *T. parva* piroplasm and infected lymphoblastoid cell DNA. After extensive hybridizations, subclones pgTpM-23 and lgTpM-58 were selected for use as species- and stock-specific *T. parva* DNA probes.

Each probe was radiolabelled with [α-^{32}P] dATP by nick translation to a specific

activity of 0.5 - 2.0 x 10^8 counts per minute (cpm) per µg DNA (Maniatis, Fritsch & Sambrook, 1982). Radiolabelled DNA was filtered through a mini-column of Sephadex G-50 in a 1x TNE solution, denatured at 100°C for 10 minutes, and added to the hybridization fluid surrounding the filters to a concentration of 1x10^6 cpm/ml fluid. Filters were incubated for an additional 14-18 hours in a 65°C shaking water bath.

After hybridization, unbound and non-specifically bound probe was removed by two washes (20-30 minutes each) in a 2x SSC, 0.1% SDS solution at 65°C. To determine the stability of the probe's hybridization to the parasite DNA, subsequent washes of higher stringency were performed for each experiment. These higher stringency washes (in solutions of 1x, and 0.5x or 0.1x SSC, 0.1% SDS) were also done at 65°C, two times for each stringency, 30-40 minutes for each wash. Autoradiographs were prepared by placing the filters under Fuji RX100 X-ray film in metal cassettes at -80°C. Exposure time periods ranged from 16 hours to one week.

Salivary glands intended for microscope examination were placed five or six glands per slide, teased apart with pins, and allowed to dry. Each slide was fixed in Carnoy's fixative, stained with methyl green/pyronin (Bancroft & Stevens, 1975), and examined for infected acini. A tick salivary gland with several infected acini is shown in Fig. 7. The stain used is in Fig. 7 is Fuelgen's stain, but the contrast between infected and uninfected acini is similar to that obtained with methyl green/pyronin.

The experimental method chosen required a comparison between the number of infected acini seen in one gland by visual examination and the number detected in the other gland of the same tick by DNA probe hybridization. To ensure that such a

comparison was valid, infection rates of both left- and right-side salivary glands were measured from five different *T. parva* stocks. Each gland was placed on a slide, teased apart, fixed, stained, and examined under the microscope for number of infected acini.

Results

The correlation between the detection of *T. parva* sporozoites by genomic hybridization and the detection of infected acini by light microscopic examination of stained salivary glands is shown in Table 1.

Probe pgTpM-23

Correlations ranged from 84% with the *T. p. parva* Mariakani stock, to 100% with the *T. p. parva* Muguga (Musoke), *T. p. parva* Uganda, and *T. p. bovis* Boleni stocks (Table 1). Non-correlating results were due to hybridization on filter without visual confirmation on microscopic examination, and visual detection without hybridization.

The probe did not hybridize to uninfected negative controls, even after one week autoradiograph exposures. Probe pgTpM-23 appeared to be capable of detecting as few as one infected acinus in a salivary gland. The intensity of the hybridization signal and size of the hybridization dot correlated roughly with the number of infected acini. The optimal washing-solution stringency and autoradiograph exposure time varied with each stock from 0.1x SSC to 2x SSC, and from 16 hours (overnight) to 72 hours (three days), respectively (Table 2). Results of hybridization to *T. p. parva* (Uganda and Muguga) and *T. p. parva* (Marikebuni and Muguga) are shown in Figures 8 and 9.

Probe lgTpM-58

Correlations ranged from 90% with *T. p. parva* Marikebuni to 100% with *T. p.*

parva Muguga (Musoke); non-correlation was due to discrepancies between visual examination and hybridization in detection of infected acini (Table 1). Optimal washing-solution stringency varied with each stock from 0.1x SSC to 1.0x SSC; the autoradiograph exposure time giving best results varied from 16 hours to one week (Table 3).

Probe lgTpM-58, unlike probe pgTpM-23, gave a faint hybridization signal to uninfected-control sailivary glands after extended exposure times (Figure 10c); however, this was not seen after short exposure times (16 hours). Like probe pgTpM-23, probe lgTpM-58 appeared to be capable of detecting as few as one infected acinus in a salivary gland, and intensity of hybridization signal was again associated with level of salivary gland infection. Autoradiograph results of hybridization to *T. p. parva* (Marikebuni) are shown in Figure 10.

Salivary gland infection rates: left vs. right

Microscopic examination of both glands from 62 infected ticks showed that in 13%, positive acini were observed in one gland and not the other; in 19%, the difference between the level of infection of paired glands was 50% or greater, using the more highly infected gland as the denominator. However, in 50%, the difference between paired glands was 20% or less, again using the more highly infected gland as the denominator (Table 4).

Table 5 shows a sample of the results used to calculate the figures listed in Table 4. The inconsistency of visual examination as the standard for judgement of tick

infection is apparent; the two observers are highly experienced technicians, but the reported number of infected acini differs between the two. In some cases, one observer identifies several infected acini where the other sees none.

Discussion

The experiments described herewith show the ability of repetitive DNA probes to specifically detect *Theileria* sporozoites within the salivary gland of the tick vector, *Rhipicephalus appendiculatus.* As a diagnostic indicator, probe pgTpM-23 had a specificity of 97.0% and a sensitivity of 94.1% at a washing stringency of 1x SSC and an autoradiograph exposure time of 16 to 72 hours. Probe lgTpM-58 had a specificity of 92.1% and a sensitivity of 91.6% at a washing stringency of 0.1x SSC and an autoradiograph exposure time of 48 to 72 hours (Table 6). These statistics change only slightly when the ranges of both wash stringencies and autoradiograph exposure times are broadened to include experiments performed under all conditions. The specificity and sensitivity of probe pgTpM-23 changes to 94.0% and 98.9%, respectively, and the specificity and sensitivity of probe lgTpM-58 become 93.5% and 90.8%, respectively. These values deviate less than 2.0% from those values found under optimal conditions.

Although the two probes have similar statistical profiles, the shorter exposure time required of pgTpM-23, combined with its complete lack of non-specific hybridization to uninfected controls, make it a more useful diagnostic probe. These characteristics may be due to the presence of more copies of the pgTpM-23 probe sequence in the *T. parva* genome, as compared to the lgTpM-58 sequence (Conrad et al., 1987b). Another possible explanation lies in the amount of non-*Theileria*-specific DNA contained in each probe: probe pgTpM-23 is inserted in plasmid vector pUC19, which has a

genome of only 2686 bp, whereas probe lgTpM-58 is packaged in phage λ gt11, with a genome of 48,516 bp, thus allowing for more non-specific hybridization.

The false positive error rate (3.0%) and false negative error rate (5.9%) of probe pgTpM-23 were within the range expected, based on the results of the right gland vs. left gland infection rate study (Table 4), in which 13% of ticks were found to be infected in one gland alone. Furthermore, the possibility of error in visual examination of the stained acini exists and must be considered, as Table 5 shows. The positive predictive value for pgTpM-23 is 96%, and that for lgTpM-58 is 98%; while these values are dependent on the prevalence of the organism in the population of ticks studied, they are presented here as realistic samples of the probes' capabilities.

Both probes appeared to be able to detect as few as one infected acinus in a salivary gland. The number of infective sporozoites in a single infected acinus is estimated at 5×10^4 but the actual numbers may vary with the parasite stock (Fawcett et al., 1982).

One aspect of the materials and methods chosen merits consideration. No attempt was made to remove RNA or protein from the nylon filters containing the processed salivary glands. One study of DNA probe hybridization to gross anatomical parts of *Drosophila* has shown that RNAse or Proteinase-K treatment does not improve results (Tchen *et al.*, 1985). However, a study hybridizing a probe specific for *Plasmodium falciparum* to lysed red blood cells required Proteinase-K digestion of the red blood cells to reduce non-specific background to acceptable levels (Barker *et al.*,

1986). High background was not a problem in the present study.

Theileria taurotragi is a less pathogenic species, carried by *Rhipicephalus spp.*, and endemic in the eland (*Taurotragus oryx*) population in many of the same areas of Africa where *T. parva* is found (Irvin, 1987). Tick salivary glands infected with *T. taurotragi* appear morphologically similar under light microscope examination to those infected with *T. parva* (Young *et al.*, 1980). In previous experiments, the probes did not hybridize to purified *T. taurotragi* piroplasm DNA after washing in a 1x SSC, 0.1% SDS solution (Conrad *et al.*, 1987b). Future experiments could determine that they are capable of distinguishing the two parasite species within tick salivary glands. Additionally, the reactivity of the probes with stocks of pathogenic *T. parva lawrencei* isolated from buffalo (*Syncercus caffer*) carriers could be investigated. Buffalo represent the major wildlife reservoir of virulent strains of *T. parva* (Conrad *et al.*, 1987a; Irvin, 1987), and therefore should be included in the testing of the probes if they are to be considered diagnostic for pathogenic *Theileria.*

A major limitation to field use of the method described in the present study is the need to dissect the tick salivary glands; this necessitates dissection equipment and trained personnel. Hybridizing the probe to intestinal fluid of infected ticks would be one way to surmount this problem. The intestines should contain parasite if the tick has fed on an infected animal within the last few days before collection, a likely situation if the ticks are pulled from the animal itself. The experimental protocol would involve puncturing or cutting the tick's body in the midsection, smearing the intestinal contents on a filter, and hybridizing the filters with the *T. parva*-specific DNA probe(s). Our initial

attempts at using this method failed due to non-specific binding of the probes to the large amounts of tick material on the filter (results not shown). However, experiments using less tick material on the filter, altering the hybridization conditions, or employing polymerase chain reaction (PCR) technology to amplify *T. parva*-specific DNA sequences in tick gut preparations might overcome these problems. Since there appears to be stock-specific variation in the pgTpM-23 repetitive sequence, amplification of sequence domains unique to a stock by PCR may be used to detect and distinguish that stock from others. PCR technology could be applied with *Theileria*-specific probes to improve the sensitivity of detecting theilerial parasites in ticks, as well as in blood and lymph node biopsy samples from infected animals. The use of these and other *Theileria* species- or stock-specific probes with non-radioactive enzyme-linked hybridization assays may also permit rapid, reliable testing of ticks for field epidemiological purposes.

The problems involved in *Theileria* control are enormous, and the solutions available today can themselves create new problems. The infection and treatment protocol provide immunity to certain forms of theileriosis in cattle, but tick spraying must be continued if other tick-borne diseases are to be held at bay. Acaricide use has many practical problems, including inadequate treatment frequency, expense, and development of tick resistance. Deforestation by overgrazing is a well-recognized threat to the land, linked to overpopulation of herds and cattle owners' assumptions that a bigger herd is always a better herd. In addition, cattle hybrids introduced into Africa to increase productivity are generally more susceptible to environmental pressures

such as tick-borne disease, trypanosomiasis, and dehydration secondary to drought; thus they are not as productive as expected. Such problems go beyond the scope of this thesis, but must be addressed if greater agricultural development of Africa is to be achieved.

References

Allsopp BA, Allsopp MT (1987) *Theileria parva:* genomic DNA studies reveal intra-specific sequence diversity. Mol Biochem Parasitol 28,77-84

Allsopp BA, Carrington M, Baylis H, Sohal S, Dolan T, Iams K (1989) Improved characterization of *Theileria parva* isolates using the polymerase chain reaction and oligonucleotide probes. Mol Biochem Parasitol 35, 137-148

Bancroft JD, Stevens A (1975) Histopathological stains and their diagnostic uses. Churchill Livingstone, Longman Inc, New York

Barker RH Jr, Suebsaeng L, Rooney W, Alecrim GC, Dourado HV, Wirth DF (1986) Specific DNA probe for the diagnosis of *Plasmodium falciparum* malaria. Science 231, 1434-1436

Barnett SF (1977) *Theileria.* In "Parasitic Protozoa" (JP Kreier, ed) Vol 4 pp 77-113. Academic Press, New York

Bhasin VK, Clayton C, Trager W, Cross AM (1985) Variations in the organization of repetitive DNA sequences in the genomes of *Plasmodium falciparum* clones. Mol Biochem Parasitol15, 149-158

Conrad PA, Stagg DA, Grootenhuis JG, Irvin AD, Newson J, Njamunggeh REG, Rossiter PB, Young AS (1987a) Isolation of *Theileria* parasites from African buffalo (*Syncerus caffer*) and characterization with anti-schizont monoclonal antibodies. Parasitol 94, 413-423

Conrad PA, Iams K, Brown WC, Sohanpal B, ole MoiYoi OK (1987b) DNA probes detect genomic diversity in *Theileria parva* stocks. Mol Biochem Parasitol 15, 149-158

Conrad PA, Baldwin CL, Brown WC, Dolan TT, Goddeeris BM, DeMartini JC, ole MoiYoi OK (1989) Infection of bovine T cell clones with genotypically distinct *Theileria parva* parasites and analysis of their cell surface phenotype. Parasitol 99, 205-213

Dolan TT, Linyonyi SK, Mbogo SK, Young AS (1984) Comparison of long acting oxytetracycline and parvaquone in immunisation against East Coast fever by infection and treatment. Res Vet Sci 44,15-20

Dolan TT (1987) Immunization to control East Coast fever. Parasitol Today 3, 4-6

Erttmann KD, Unnasch TR, Greene BM, Albiez EJ, Boateng J, Denke AM, Ferraroni JJ, Karam M, Schulz-Key H, Williams PN (1987) A DNA sequence specific for forest-form *Onchocerca volvulus*. Nature 327, 415-417

Fawcett DW, Buscher G, Doxsey S (1982) Salivary gland of the tick vector of East Coast Fever. III. The ultrastructure of sporogeny in *Theileria parva*. Tissue Cell 14, 183-206

Franzen L, Shabo R, Perlmann H, Wizgell H, Westin G, Aslund L, Persson T, Pettersson U (1984) Analysis of clinical specimens by hybridisation with probe containing repetitive DNA from *Plasmodium falciparum*. Lancet 257, 525-528

Irvin AD, Dobbelaere DAE, Mwamachi DM, Minami T, Spooner PR, Ocama JGR (1983) Immunisation against East Coast fever: correlation between monoclonal antibody profiles of *Theileria parva* stocks and cross immunity *in vivo*. Res Vet Sci 35, 341-346

Irvin AD (1987) Characterization of Species and Strains of *Theileria*. Adv Parasitol 26, 145-197

Jackson PR, Lawrie JM, Stiteler JM, Hawkins DW, Wohlhieter JA, Rowton ED (1986) Detection and characterization of *Leishmania* species and strains from mammals and vectors by hybridization and restriction endonuclease digestion of kinetoplast DNA. Vet Parasitol 20, 195-215

Jelinek WR, Schmid CW (1982) Repetitive sequences in eukaryotic DNA and their

expression. Annu Rev Biochem 51, 813-844

Kukla BA, Majiwa PAO, Young JR, Moloo SK, ole MoiYoi OK (1987) Use of species-specific DNA probes for detection and identification of trypanosome infection in tsetse flies. Parasitol 95, 1-16

Maniatis F, Fritsch EC, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

McLaughlin GL, Edlind TD, Ihler GM (1986) Detection of *Babesia bovis* using DNA hybridization. J Protozool 1986, 125-128

McReynolds LA, DeSimone SM, Williams SA (1986) Cloning and comparison of repeated DNA sequences from the human filarial parasite *Brugia malayi* and the animal parasite *Brugia pahangi*. PNAS 83, 797-801

Melhorn H, Schein E (1984) The piroplasms: life cycle and sexual stages. Adv Parasitol 23, 37-103

Miller LH, Pino JA, McKelvey JJ Jr (1977) Immunity to blood parasites of animals and man. Plenum Press, New York

Morel C, Chiari E, Camargo EP, Mattei DM, Romanha AJ, Simpson L (1980) Strains and clones of *Trypanosoma cruzi* can be characterized by pattern of restriction endonuclease products of kinetoplast DNA minicircles. PNAS 77, 6810-6814

Peacock WJ, Lohe AR, Gerlach WL, Dornsmuir P, Dennis ES, Appels R (1977) Fine structure and evolution of DNA in heterochromatin. Cold Spring Harbor Symp Quant Biol 42, 1121-1135

Perler FB, Karam M (1986) Cloning and characterization of two *Onchocerca volvulus* repeated DNA sequences. Mol Biochem Parasitol 21, 171-178

Pollack Y, Metzger S, Shemer R, Landau D, Spira DT, Golenser J (1985) Detection of *Plasmodium falciparum* in blood using DNA hybridization. Am J Trop Med Hyg 34, 663-667

Radley DE, Brown CGD, Burridge MJ, Cunningham MP, Kirimi IM, Purnell RE, Young AS (1975) East Coast fever. I. Chemoprophylactic immunization of cattle against *Theileria parva* (Muguga) and five theilerial strains. Vet Parasitol 1, 35-41

Rogers WO, Burnheim PF, Wirth DF (1988) Detection of *Leishmania* within sand flies by kinetoplast DNA hybridization. Am J Trop Med Hyg 39, 434-439

Sim BKL, Mak JW, Cheong WH, Sutanto I, Kurniawan L, Marwoto HA, Franke E, Campell JR, Wirth DF, Piessens WF (1986) Identification of *Brugia malayi* in vectors with a species-specific DNA probe. Am J Trop Med Hyg 35, 559-564

Sturm NR, Degrave W, Morel C, Simpson L (1989) Sensitive detection and schizodeme classification of Trypanosoma cruzi cells by amplification of kinetoplast minicircle DNA sequences: Use in diagnosis of Chagas' disease. Mol Biochem Parasitol 33, 205-214

Tchen P, Anxolabehere D, Nouaud D, Periquet G (1985) Hybridization on squashed flies: a method to detect gene sequences in individual drosophila. Analyt Biochem 150, 415-420

Uilenberg G (1981) Theilerial species of domestic livestock. In "Advances in the Control of Theileriosis" (AD Irvin, MP Cunningham and AS Young, eds) pp 4-37. Martinus Nijhoff Publishers, The Hague

van Eys GJ, Schoone GJ, Ligthart GS, Alvar J, Evans DA, Terpstra WJ (1989) Identification of 'Old World' *Leishmania* by DNA recombinant probes. Mol Biochem Parasitol 34, 53-62

Wirth DF, Pratt DM (1982) Rapid identification of *Leishmania* species by specific

hybridization of kinetoplast DNA in cutaneous lesions. PNAS 79, 6999-7003

World Health Organization (1978) Proposals for the nomenclature of salivarian trypanosomes and for the maintenance of reference collections. Bull World Health Organ 56, 467-480

Young AS, Grootenhuis JO, Leitch BV, Schein E (1980) The development of *Theileria (cytauxzon) taurotragi* (Martin and Brocklesby, 1960) from eland in its tick vector *Rhipicephalus appendiculatus*. Parasitol 81, 129-144

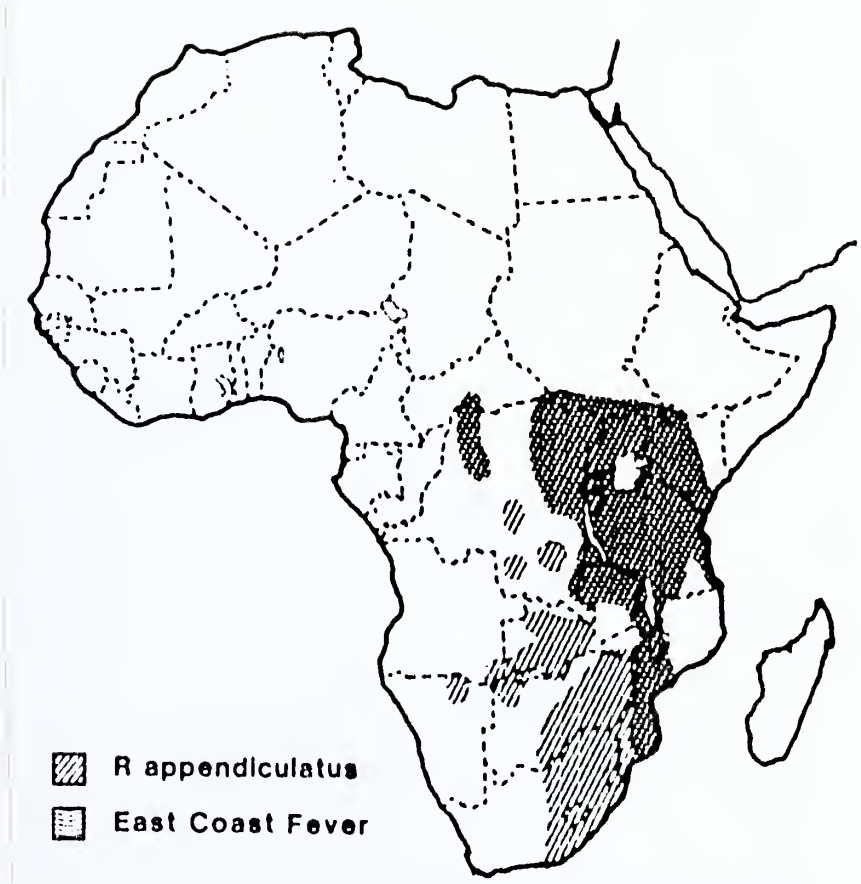


Figure 1. Distribution of East Coast fever (reproduced with permission from Dolan, 1987)

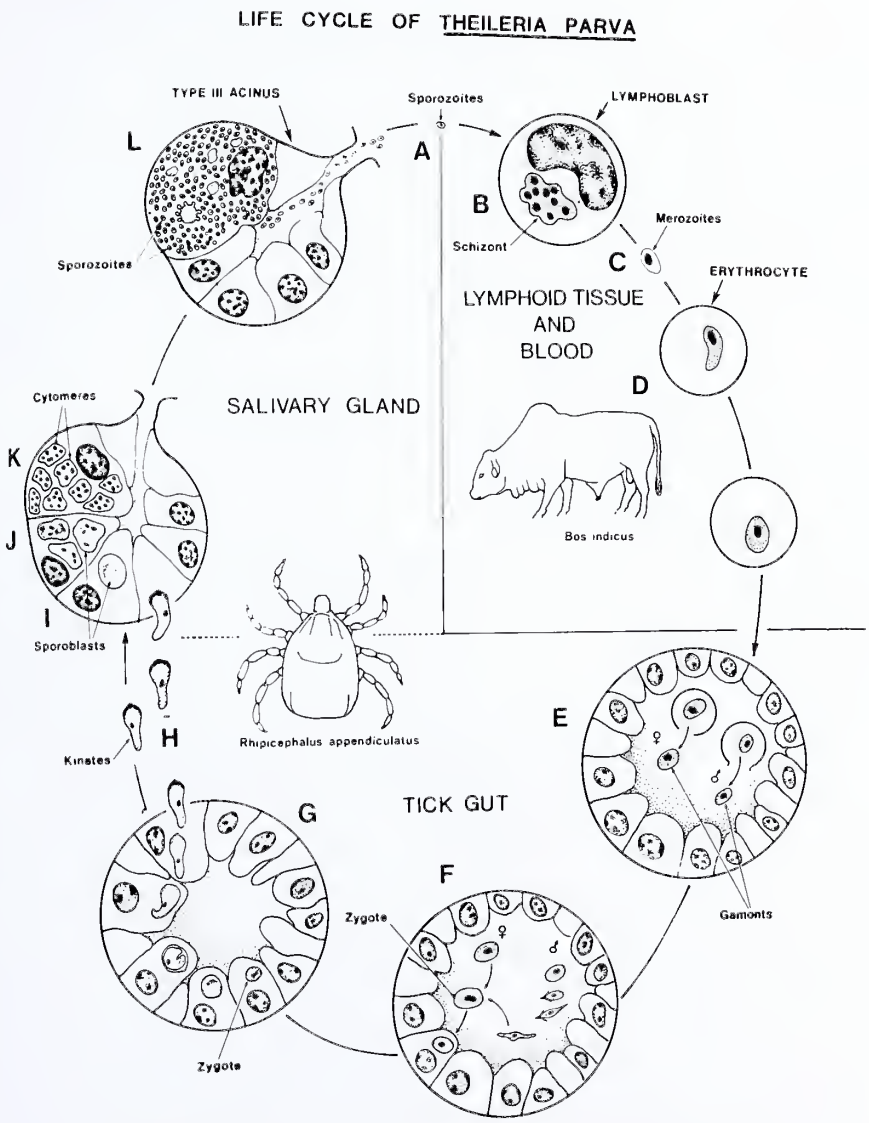


Fig. 1. Schematic representation of the life cycle of *Theileria parva*. Composite based upon diagrams by Schein and Friedhoff (1978), Mehlhorn *et al.* (1979) and Young *et al.* (1980). For explanation see text.

Figure 2. Life cycle of *Theileria* (reproduced with permission from Fawcett, Buscher & Sambrook, 1982)

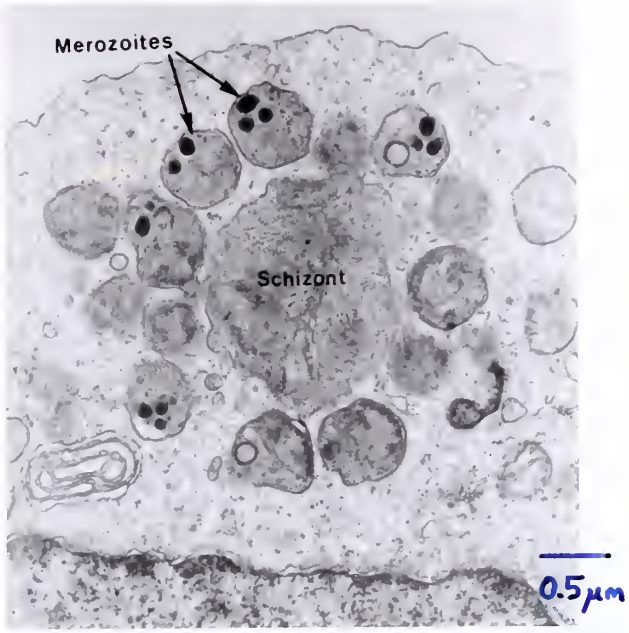


Figure 3. Electron micrograph of bovine lymphocyte infected with *Theileria* schizonts (courtesy of Dr. Donald Fawcett)

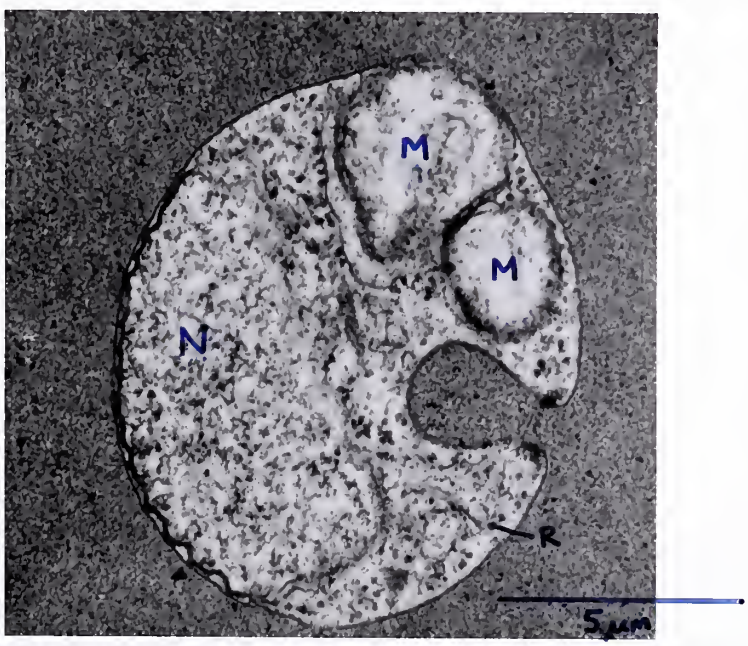


Figure 4. Electron micrograph of piroplasm stage of *Theileria*. N=nucleus, M=mitochondria, R=ribosome. (Courtesy of Dr. Patricia Conrad)

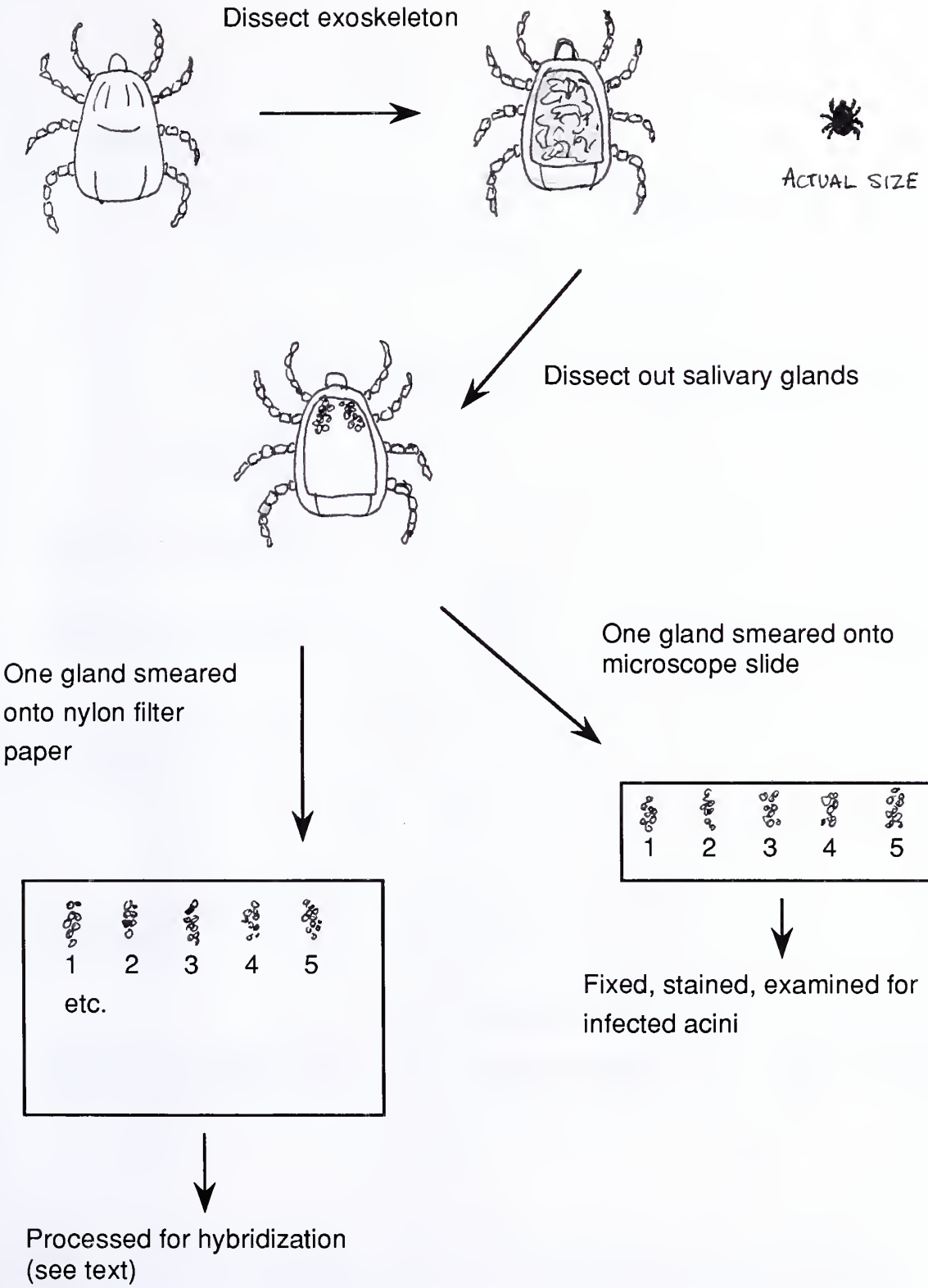
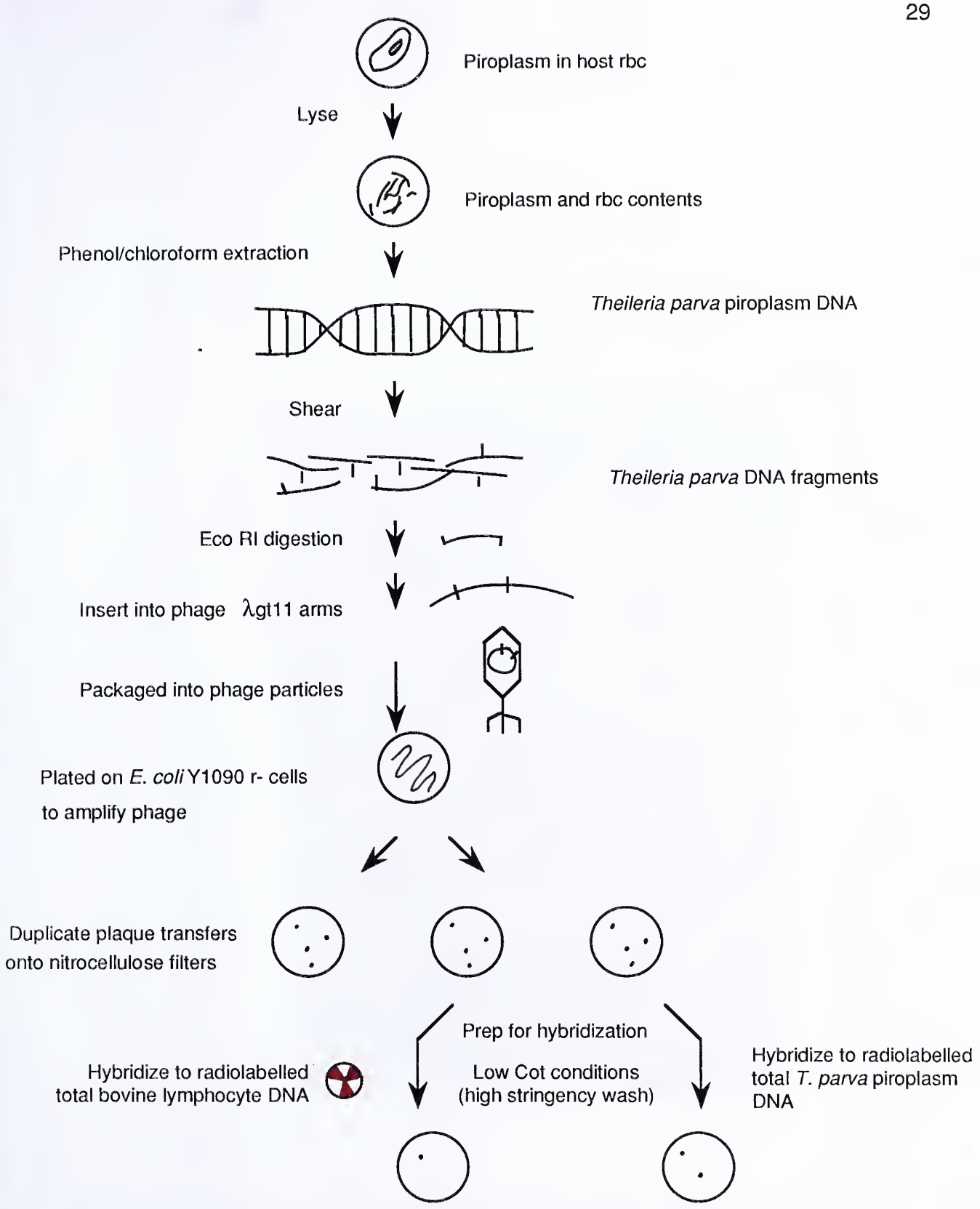


Fig. 5. *R. appendiculatus* (brown ear tick) dissection.



Select plaques that contain high amounts of repetitive DNA and thus will hybridize strongly to *T. parva* DNA but contain no bovine DNA and so will not hybridize to total bovine lymphocyte DNA

To purify, use selected plaque(s); repeat plating on *E. coli,* plaque transfers, and hybridizations x 2

Figure 6. Development of *Theileria parva*-specific DNA probe

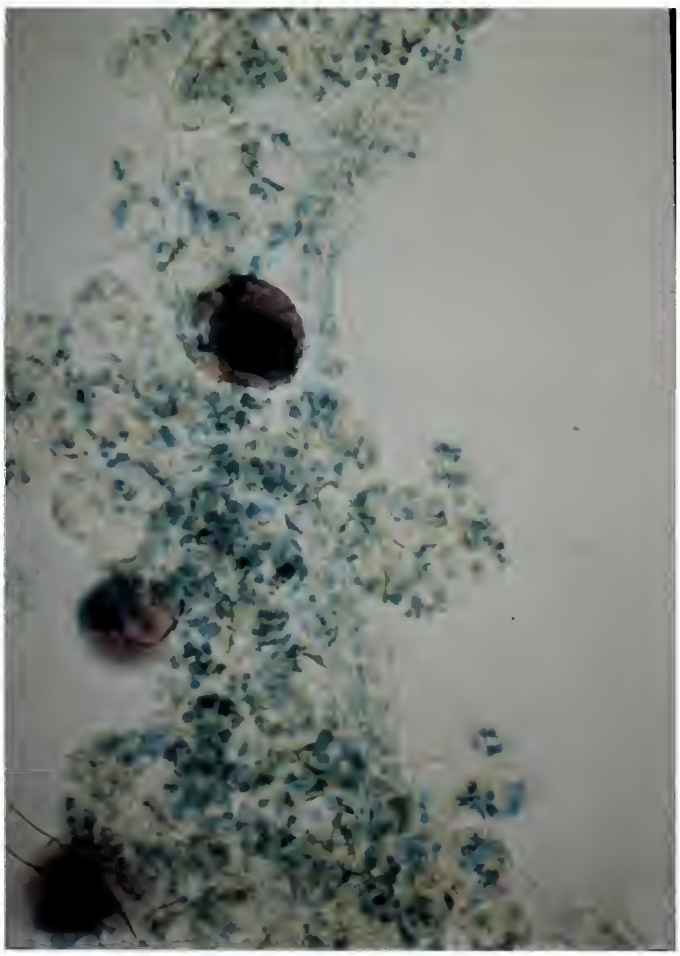


Figure 7. Light micrograph of salivary gland acini stained with Fuelgen's stain. Infected acini contain purple-stained sporozoites. (Courtesy of ILRAD)

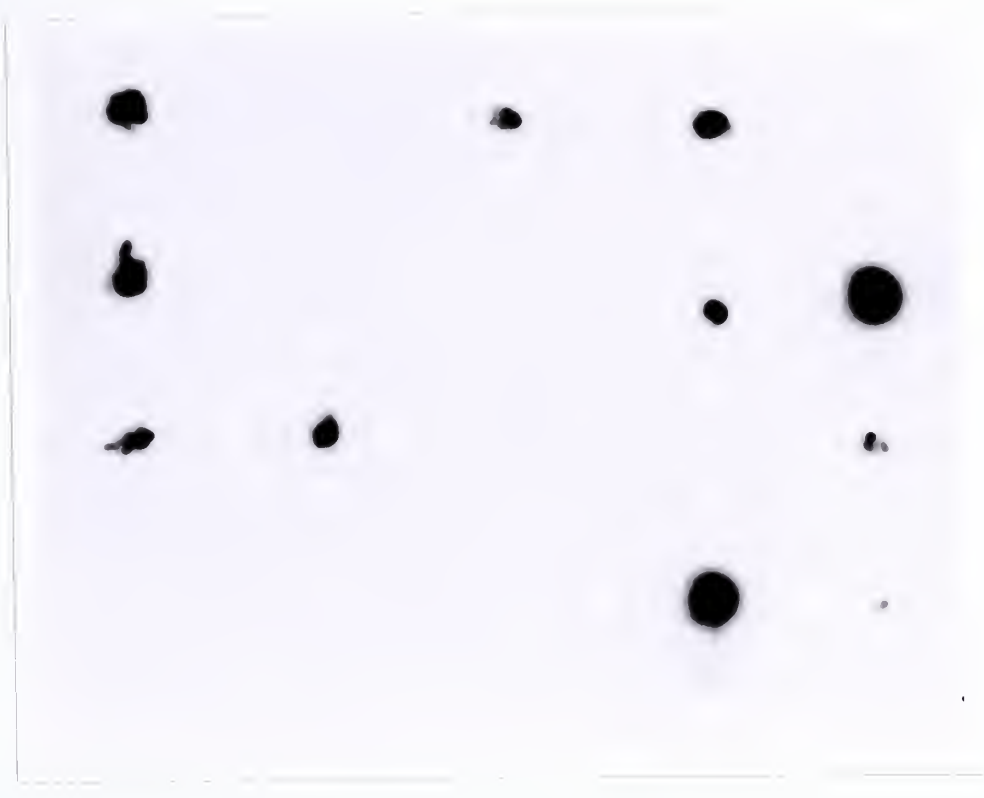


Figure 8a. Autoradiograph of hybridization of probe pgTpM-23 to *T. p. parva* (Uganda) and *T. p. parva* (Muguga). Wash stringency 1x SSC, autoradiograph exposure time 16 hours.

T. parva (Uganda)	49	0	19	38	0
T. parva (Muguga)	1	0	0	1	39
T. parva (Uganda)	19	35	0	0	17
Uninfected	0	0	0		
T. parva (Muguga) (positive control)				45	21

Figure 8b. Number of infected acini in the corresponding salivary gland as detected by light microscopic examination

Figure 9a. Autoradiograph of hybridization of probe pgTpM-23 to *T. p. parva* (Marikebuni) and *T. p. parva* (Muguga). Wash stringency 1x SSC, autoradiograph exposure time 16 hours.

T. parva (Marikebuni)	12	0	0	40	2
T. parva (Muguga)	0	53	19	1	55
T. parva (Marikebuni)	13	43	122	38	0
Uninfected	0	0	0		
T. parva (Muguga) (positive control)				8	1

Figure 9b. Number of infected acini in the corresponding salivary gland as detected by light microscopic examination

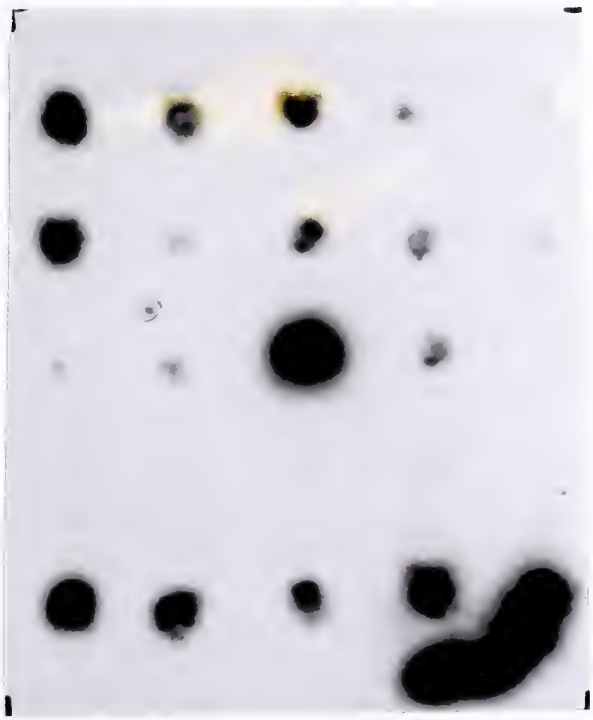


Figure 10a. Autoradiograph of hybridization of probe lgTpM-58 to *T. p. parva* (Marikebuni) and *T. p. parva* (Muguga). Wash stringency 0.1x SSC, autoradiograph exposure time 72 hours.

T. parva (Marikebuni)	55	51	49	6	0
T. parva (Marikebuni)	125	13	70	4	3
T. parva (Marikebuni)	3	0	3	4	21
Uninfected	0	0	0	0	0
T. parva (Muguga) (positive control)	+	+	+	+	+

Figure 10b. Number of infected acini in the corresponding salivary gland as detected by light microscopic examination

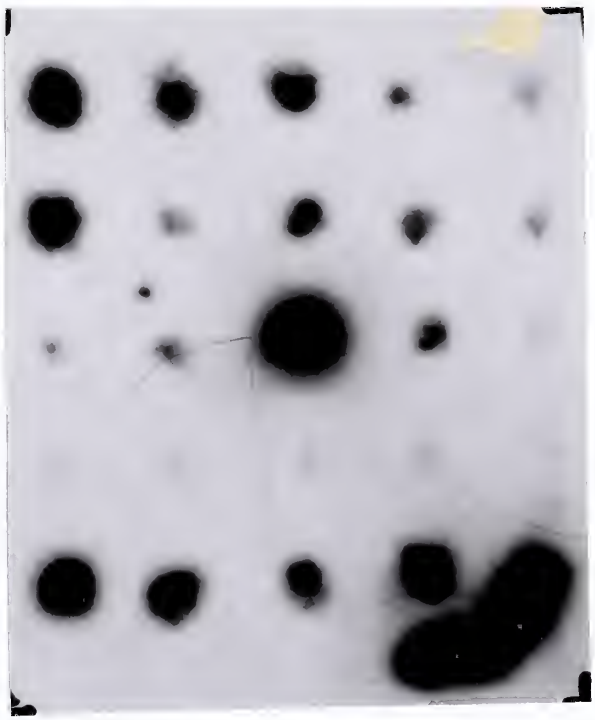


Figure 10c. Autoradiograph of hybridization of probe lgTpM-58 to *T. p. parva* (Marikebuni) and *T. p. parva* (Muguga). Same experiment as Fig. 10a, but autoradiograph exposed for seven days. Note faint hybridization signal from uninfected controls.

Table 1. Correlation between visual examination and hybridization of probes pgTpM-23 and lgTpM-58 in detection of *Theileria parva* in tick salivary glands

		pgTpM 23		lgTpM 58	
T. parva Stock	Stab [1]	%[2]	*N* [3]	%	*N*
Muguga (bulk)	1587	93.2	44	92.0	37
Muguga (Musoke)[4]	1004	100.0	58	100.0	40
Uganda	1676	100.0	39	91.1	45
Marikebuni	2245	92.7	55	90.0	60
Zanzibar (south)	2914	92.0	25	92.0	25
Mariakani	1937	84.0	25	96.0	25
Boleni	2628	100.0	200	--	NT[5]
Uninfected	----	100.0	108	100.0[6]	87

[1] Stab = number of cryopreserved stabilate used to infect calves

[2] % = % correlation between visual examination and DNA hybridization

[3] *N* = total number of ticks tested

[4] positive control

[5] NT = not tested

[6] see text

Table 2. Results of hybridization of probe pgTpM-23 to tick salivary glands

Expt.	Stringency(SSC)[1]	Exposures [2]	Stock [3]	Opt String [4]	Opt Exp [5]	C/T [6]
1	0.1x	30h, 1w	*Tp* Mug (b)	0.1x	all	12/12
2	0.1x	16h, 1w	*Tp* Mug (b)	0.1x	16h	9/12
3	1x/ 0.1x	16, 48h/ 72h, 1w	*Tp* Mug (b)	all	all	5/5
			Tp Uganda	0.1x	72h	9/9
4	1x/ 0.5x	16, 48, 72h/ 1w	*Tp* Mug (b)	all	all	5/5
			Tp Mrkbuni	1x	16h	10/10
5	2x/ 1x/ 0.1x	16, 72h/ 96h/ 1w	*Tp* Zanz (s)	1x	96h	9/10
			Tp Markani	2x/ 1x	16h/ 96h	9/10
6	2x/ 1x	16, 72h/ 1w	*Tb* Boleni	2x	16h	200/200
7	1x	16, 72h, 1w	*Tp* Uganda	1x	all	20/20
			Tp Mrkbuni	1x	16, 72h	18/20
8	1x	16, 72h, 1w	*Tp* Mrkbuni	1x	72h, 1w	14/15
9	1x	16, 72h, 1w	*Tp* Zanz (s)	1x	72h, 1w	14/15
			Tp Markani	1x	1w	13/15
10	1x	16, 72h, 1w	*Tp* Mug (b)	1x	16h	10/10
			Tp Uganda	1x	16, 72h	10/10
			Tp Mrkbuni	1x	16h	9/10

[1] stringency of washing solution (SSC) at which autoradiographs were prepared

[2] autoradiograph exposure times; h = hours, w = week

[3] *Tp* = *Theileria parva parva*, *Tb* = *Theileria parva bovis*, Mug (b) = Muguga (bulk), Mrkbuni = Marikebuni, Zanz (s) = Zanzibar (south), Markani = Mariakani

[4] Opt String = stringency of washing solution (SSC) at which optimal results were obtained

[5] Opt Exp = autoradiograph exposure times at which optimal results were obtained

[6] C/T = number correlating/ total number, at optimal stringency and optimal exposure time

Table 3. Results of hybridization of probe lgTpM-58 to tick salivary glands

Expt.	Stringency(SSC)[1]	Exposures[2]	Stock[3]	Opt String[4]	Opt Exp[5]	C/T[6]
1	0.1x	16h, 1w	*Tp* Mug (b)	0.1x	1w	12/12
2	0.1x	16, 48h	*Tp* Mug (b)	0.1x	16h	5/5
			Tp Uganda	0.1x	all	5/5
			Tp Mrkbuni	0.1x	48h	5/5
3	2x/ 1x/ 0.1x	16, 72h/ 96h/ 1w	*Tp* Zanz (s)	2x	16h	10/10
			Tp Markani	2x/ 1x	16h/ 72h	9/10
4	0.1x	16, 72h, 1w	*Tp* Uganda	0.1x	72h, 1w	28/30
			Tp Mrkbuni	0.1x	72h	28/30
5	0.1x	16, 72h, 1w	*Tp* Mrkbuni	0.1x	16, 72h	13/15
6	1x/ 0.1x	16, 72h/ 1w	*Tp* Zanz (s)	all	all	13/15
			Tp Markani	all	all	15/15
7	0.1x	16, 72h, 1w	*Tp* Mug (b)	0.1x	1w	19/20
			Tp Uganda	0.1x	72h	9/10
			Tp Mrkbuni	0.1x	72h, 1w	9/10

[1] stringency of washing solution (SSC) at which autoradiographs were prepared

[2] autoradiograph exposure times; h = hours, w = week

[3] *Tp* = *Theileria parva parva*, Mug (b) = Muguga (bulk), Mrkbuni = Marikebuni, Zanz (s) = Zanzibar (south), Markani = Mariakani

[4] Opt String = stringency of washing solution (SSC) at which optimal results were obtained

[5] Opt Exp = autoradiograph exposure times at which optimal results were obtained

[6] C/T = number correlating/ total number, at optimal stringency and optimal exposure time

Table 4. Summary of comparison of salivary gland infection rates

Stock	*N* [1]	+/- [2]	≥ 50% Diff [3]	≤ 20% Diff [4]
Muguga (bulk)	11	2	1	8
Marikebuni	33	4	6	15
Uganda	5	0	0	3
Zanzibar (south)	4	0	2	2
Mariakani	9	2	3	3
Total	62	8	12	31
%		13	19	50

[1] *N* = total number of ticks examined

[2] No. +/- = number of ticks with only one infected gland

[3] ≥ 50% Diff = number of ticks with ≥50% difference in number of infected acini between glands

[4] ≤ 20% Diff = number of ticks with ≤20% difference in number of infected acini between glands

Table 5. Sample of results from visual examination of stained left and right salivary gland acini

	Left salivary gland		Right salivary gland	
	Obs 1[1]	Obs 2	Obs 1	Obs 2
Tick 1	0	0	0	5
2	53	59	54	65
3	17	17	13	13
4	2	4	0	1
5	4	6	0	3
6	25	15	28	25
7	54	73	51	47
8	18	19	9	10

[1] Obs = observer

Table 6 Statistical analysis of hybridization results with *T. parva*-specific DNA probes and tick salivary glands

pgTpM-23

		Stain +	Stain -	
Probe	+	95[1] (142)[2]	2 (3)	Sensitivity = 94.1% (94.0%) Specificity = 97.0% (98.9%)
	-	6 (9)	65 (28)	false positive rate = 3.0% false negative rate = 5.9% positive predictive value = 0.96

[1] Conditions: wash stringency 1x SSC, autoradiograph exposure time 16 - 72 hours

[2] In addition to the above experiments, also includes experiments with: (1) 1x SSC wash, at any autoradiograph exposure time, and (2) 16 - 72 hours autoradiograph exposure time, at any wash stringency if no 1x SSC wash done

lgTpM-58

		Stain +	Stain -	
Probe	+	76[1] (115)[2]	5 (9)	Sensitivity = 91.6% (93.5%) Specificity = 92.1% (90.8%)
	-	7 (8)	58 (89)	false positive rate = 7.9% false negative rate = 8.4% positive predictive value = 0.98

[1] Conditions: wash stringency 0.1x SSC, autoradiograph exposure time 48 - 72 hours

[2] In addition to the above experiments, also includes experiments with: (1) wash stringency of 0.1x SSC, at any autoradiograph exposure, and (2) autoradiograph exposure of 72 hrs - one week, at any wash stringency if no 0.1x SSC wash done

HARVEY CUSHING / JOHN HAY WHITNEY
MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS DATE